A Specific Mutation in *Muc2* Determines Early Dysbiosis in Colitis-Prone *Winnie* Mice

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Background: Inflammatory bowel disease (IBD), including Crohn disease (CD) and ulcerative colitis (UC), is a multifactorial disorder characterized by chronic inflammation and altered gut barrier function. Dysbiosis, a condition defined by dysregulation of the gut microbiome, has been reported in patients with IBD and in experimental models of colitis. Although several factors have been implicated in directly affecting gut microbial composition, the genetic determinants impacting intestinal dysbiosis in IBD remain relatively unknown.

Methods: We compared the microbiome of normal, uninflamed wild-type (WT) mice with that of a murine model of UC (ie, *Winnie* strain). *Winnie* mice possess a missense mutation in *Muc2* that manifests in altered mucus production as early as 4 weeks of age, with ensuing colonic inflammation. To better address the potential role of mutant *Muc2* in promoting dysbiosis in *Winnie* mice, we evaluated homozygous mutant mice (*Winnie*$^{-/-}$) with their WT littermates that, after weaning from common mothers, were caged separately according to genotype. Histologic and inflammatory status were assessed over time, along with changes in their respective microbiome compositions.

Results: Dysbiosis in *Winnie* mice was already established at 4 weeks of age, before histologic evidence of gut inflammatory changes, in which microbial communities diverged from that derived from their mothers. Furthermore, dysbiosis persisted until 12 weeks of age, with peak differences in microbiome composition observed between *Winnie* and WT mice at 8 weeks of age. The relative abundance of Bacteroidetes was greater in *Winnie* compared with WT mice. Verrucomicrobia was detected at the highest relative levels in 4-week-old *Winnie* mice; in particular, *Akkermansia muciniphila* was among the most abundant species found at 4 weeks of age.

Conclusions: Our results demonstrate that mutant genetic determinants involved in the complex regulation of intestinal homeostasis, such as that observed in *Winnie* mice, are able to promote early gut dysbiosis that is independent from maternal microbial transfer, including breastfeeding. Our data provide evidence for intestinal dysbiosis attributed to a *Muc2*-driven mucus defect that leads to colonic inflammation and may represent an important target for the design of future interventional studies.

Key Words: microbiome, inflammatory bowel disease (IBD), dysbiosis, murine model of ulcerative colitis (UC)

INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn disease (CD) and ulcerative colitis (UC), are multifactorial disorders characterized by chronic bouts of inflammation and remission of the gastrointestinal tract that symptomatically present with abdominal pain, diarrhea, and rectal bleeding.[1, 2]

Although the precise etiology is currently unknown, it is widely accepted that IBD results from a multifactorial

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combination of genetic predisposition and environmental factors.[1, 3–5] In the 20th century, increased urbanization, hygiene practices, air pollution, and widespread use of antibiotics have been reported to contribute to the onset of IBD, particularly in industrialized countries.[6–8] Epidemiological studies suggest that diets characterized by a high content of animal protein and fats and low fiber and antioxidants sustain the prevalence of bacteria with proteolytic and putrefactive metabolism, particularly on saccharolytic bacteria. Proteolytic bacteria produce toxic metabolites, such as thiols, phenols, indoles, amines, and branched-chain fatty acids, which can lead to reduction of the inner mucus layer, intestinal barrier impairment, loss of immune tolerance, and perturbation of normal gut homeostasis.[6, 9–11]

Under homeostatic conditions, a dense layer of mucus separates luminal microbial communities from the host's enterocytes.[12] While the inner mucus layer is inaccessible to the vast majority of bacterial species, an important interface exists between the gut microbiome and the outer mucus barrier that represent their habitat.

It is well established that the intestinal microbiome is of paramount importance in the maintenance of gut health. In fact, recent studies underscore its importance for normal intestinal homeostasis by maintaining symbiosis between host and microbial communities that can regulate the fine equilibrium between tolerance and inflammation. However, to date, it is still unclear whether the shift from a "healthy microbiome" to a state of "intestinal dysbiosis" is the result of a chronically inflamed intestine or if dysbiosis triggers gut dysfunction, including dysregulated intestinal permeability and immune response.[13–15]

In general, dysbiosis in IBD patients is characterized by a reduction in microbial diversity compared with healthy patients and an overgrowth of specific bacterial species that are usually underrepresented.[13] Importantly, dysbiosis persists even in IBD patients who respond to pharmacological intervention and achieve remission;[16] in fact, it has been proposed that persistent dysbiosis may be the trigger for disease recurrence in these patients. Therefore, it is important to utilize experimental models that mirror the spontaneous and progressive nature of IBD to address the precise timing and specific role(s) of dysbiosis and its onset.

Herein, we evaluated the gut microbiome in a spontaneous model of UC called *Winnie* and compared that derived from wild-type (WT) mice from 4 to 16 weeks. Both mice strains were obtained as littermates of same *Winnie*+/- parents. In *Winnie* mice, the missense mutation of the *Muc2* gene causes aberrant MUC2 synthesis and depletion of the mucus layer, resembling that of UC patients.[17] We performed a detailed evaluation of the microbiome and colonic pathology of *Winnie* and WT control littermates, from weaning to adult age. Our results show that, in contrast to what has previously been described, the onset of specific microbial communities is detectable in 4-week-old *Winnie* mice, even before the emergence of the intestinal UC-like morphologic features that are characteristic of this strain. Our data indicate that the composition of the gut microbiome is determined by mouse genotype before the onset of colitis, suggesting that dysbiosis observed in the *Winnie* strain is likely a trigger, more than a consequence, of chronic colonic inflammation in these mice.

METHODS

Ethical Considerations

Our studies were conducted in accordance with national and international guidelines and were approved by the authors' institutional review board (Organism For Animal Wellbeing [OPBA]). All animal experiments were carried out in accordance with Directive 86/609 EEC, enforced by Italian D.L. n. 116/1992, and approved by the Committee on the Ethics of Animal Experiments of Ministero della Salute–Direzione Generale Sanità Animale (Prot. 768/2015-PR 27/07/2015) and the official RBM veterinarian. Animals were immediately killed if found to be in severe distress during the experimental period to avoid undue suffering.

Mice

C57BL/6 mice were originally purchased from Jackson Laboratories (C57BL/6, Stock No.: 000664), whereas *Winnie*$^{-/-}$ mice were obtained from the University of Tasmania (Dr. R. Eri's laboratory). These mouse strains were initially used to generate heterozygote *Winnie*$^{+/-}$ breeders. For the present study, the breeding strategy was based on co-housing heterozygote breeders to obtain both *Winnie*$^{-/-}$ and WT littermates from the same mother. Newborn pups were weaned at 4 weeks of age, ear-tagged, and then single-caged based on sex and similar genotype(s) for fecal material collection. Genotype was performed from DNA obtained from 5-mm tail tissues. Body weight, stool consistency, and rectal bleeding were assessed every 4 weeks.

Mice were killed at 16 weeks of age, and colons were removed to evaluate the clinical severity of disease. Colon lengths and weights were measured as indicators of colonic inflammation. Colon/body weight indices were calculated as the ratio of colon wet weight to total body weight (BW) and as the ratio of colon length to total BW of each mouse. Disease Activity Index (DAI) was determined by scoring changes in body weight (0–4), stool consistency (0–4), and occult blood (0–4), as previously described.[18]

Histologic Assessment

Tissue sections from the distal colon were fixed in 10% buffered formalin and embedded in paraffin. Microtome sections of 3 μm were cut and stained using a hematoxylin and eosin

standard protocol. Images were acquired using a Leica LMD 6500 microscope (Leica Microsystems, Wetzlar, Germany).

Single-Cell Preparation From Intestinal Lamina Propria and Cytofluorimetric Assay

The intestines of 16-week-old *Winnie* and WT mice were removed and tissue digested to obtain a single-cell preparation. Colons were cut into small segments (1 cm long), washed with DPBS 1X (Gibco, Waltham, MA, USA) + 2.5 mM EDTA (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) to remove epithelial cells, and digested with collagenase type IV and DNase I (Sigma Aldrich, St. Louis, MO, USA) using the GentleMacs suggested protocol for 30 minutes at 37°C. Resulting single-cell suspensions from colonic lamina propria (LP) were pelleted by centrifugation, washed with DPBS 1X + 0.5 mM EDTA, and passed through 100-µm and 30-µm cell strainers (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then washed with DPBS 1X + 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) and labeled with CD45.2-FITC and MHC II-APC (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Finally, cell suspensions were labeled with 7-AAD Staining Solution (Miltenyi Biotec, Bergisch Gladbach, Germany) to distinguish between viable and dead cells, also according to the manufacturer's instructions. Flow cytometer data analysis was performed using NAVIOS software (Beckman Coulter, Brea, CA, USA), with at least 3 experiments performed.

RNA Extraction and Quantitative Polymercase Chain Reaction Analysis

Total RNA was isolated from colons of WT and *Winnie* mice. RNA was extracted using TRIzol (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed using an iScript cDNA Synthesis kit (Biorad, CA, USA) with random primers for cDNA synthesis. Gene expression of *Ptprc* (CD45) and *Gapdh* was assessed using the TaqMan gene expression assay (Thermo Fisher Scientific, MA, USA) murine probes Mm01293577_m1 and Mm99999915_g1, respectively. Real-time analysis was performed on a CFX96 System (Biorad, CA, USA), and relative expression was calculated using the ΔΔCt method. At least 3 experiments were performed.

DNA Extraction From Fecal Material

Total genomic bacterial DNA was isolated from frozen stool samples of 4-, 8-, and 16-week-old WT and *Winnie* mice (4 samples/genotype for each time point) using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

16S rRNA Metagenetics of Gut Microbiome

16S metagenomics was carried out at Genomix4life (Salerno, Italy) using the Illumina MiSeq platform. The V3-V4 region of 16S bacterial rRNA was amplified for analysis of diversity.[19] Polymerase chain reaction (PCR) and sequencing analyses were carried out according to standard protocols of Genomix4life. Quality control (QC) and taxonomic assignments were performed according to the QIIME and Ribosomal Database Project Bayesian classifier in combination with a set of custom-designed informatic pipelines implemented by Genomix4life for analyses of microbial communities. Taxonomic attribution was carried out using the BLAST search in the NCBI 16S ribosomal RNA sequences database.[20] The percentage of each bacterial OTU was analyzed individually for each sample, providing relative abundance information among the samples based on the relative numbers of reads within each sample.[21] Alpha-diversity indexes were evaluated using the number of OTUs, Chao1 species richness, and the Shannon index. Alpha diversity was calculated using Qiime.[22, 23]

Statistical Analysis

All data are expressed as the mean ± SEM, with results obtained from 3 independent experiments. Metagenomic data (Unifrac distance metric and taxonomic abundance) were analyzed by principal component analysis (PCA)[24] using the statistical software Statistica for Windows (Statistica 6.0 for Windows 1998, StatSoft, Vigonza, Italia). Permut-MatrixEN software was used to identify clusters at the level of mouse groups and taxa.[25] Statistical analysis of relative abundance of microbial genera was based on Duncan's Multiple Range test, with a significance level of $P \leq 0.05$. Finally, unless specifically described, other data and group differences were analyzed and compared by paired or unpaired 2-tailed Student *t* tests.

RESULTS

Body Weight Loss During Colitis Depends on Genotype Predisposed to Develop UC-Like Symptoms

Experimental mice were obtained from heterozygote breeders (*Winnie*$^{+/-}$) to obtain *Winnie*$^{-/-}$ and WT mice from the same breeding pair(s). Weight differences were associated with the defect in *Muc2* that characterizes *Winnie* mice as early as 4 weeks of age, which persisted over the course of time up to 16 weeks of age. Although WT mice consistently gained weight over the course of the experimental period (~50% of the initial body weight from 4 to 16 weeks), both *Winnie*$^{-/-}$ males and females gained less weight than their WT counterparts, reaching 23.2 g in males and 18.9 g in females from 4 to 16 weeks of age (Fig. 1).

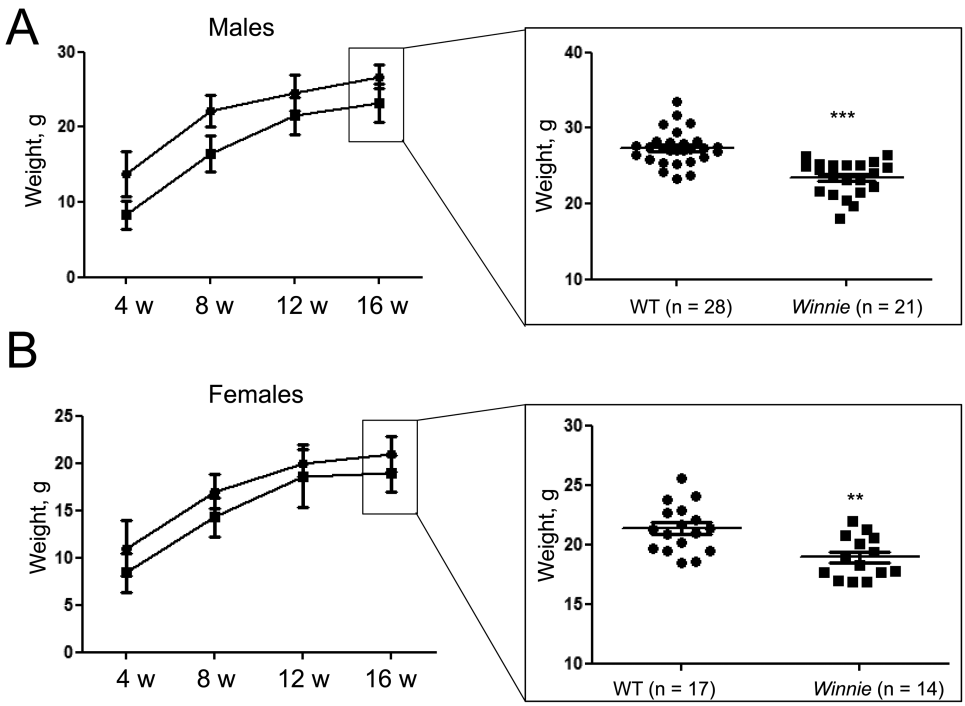


FIGURE 1. Total body weight of *Winnie* mice is consistently decreased compared with age- and sex-matched WT mice. Weight of *Winnie* and age-matched WT mice from 4 to 16 weeks in male (A) and female (B) mice. Inset graphs (right panels) depict differences in individual mice, comparing 16-week-old WT (●) and *Winnie* (▪) mice. $^{***}P < 0.001$; $^{**}P < 0.01$ using unpaired 2-tailed Student *t* tests.

Moderate Inflammation in the Intestine of 16-Week-Old *Winnie* Mice

We next compared the inflammatory status of WT and *Winnie* littermates obtained from the same breeders. As observed in previous studies, weight loss in 16-week-old *Winnie* mice correlated with the presence and severity of colonic inflammation.[17, 26] Loss of stool consistency resulting in more watery stools was also observed only in *Winnie* mice (Fig. 2A), which also showed a reduction in colon length, although this did not reach statistical significance (Fig. 2B). The colon weights of *Winnie* mice were significantly increased compared with age-matched WT mice (Fig. 2C), suggesting the presence of inflammation. Consequently, colon length/mouse weight and colon weight/mouse weight indices were also significantly increased when comparing *Winnie* with WT mice (Fig. 2D and E, respectively). Histologic evaluation of intestinal tissues confirmed the presence of moderate colitis, with epithelial erosions and immune cell infiltration in 16-week-old *Winnie* mice, as previously reported (Fig. 2F).[26] Instead, no morphologic signs of inflammation were detected in 4- and 8-week-old *Winnie* mice, which exhibited normal histology (Fig. 2F), similar to what we have previously described.[27] In addition, DAI was higher in *Winnie* mice compared with WT (5 vs 0, respectively; data not shown), with a similar trend observed when comparing female mice (3 WT and 4 *Winnie*; data not shown). As no significant differences were observed between male and female mice, we restricted our analyses to male mice in subsequent experiments, which were single-caged to avoid coprophagy.

Increased Immune Cell Infiltration in LP of 16-Week-Old *Winnie* Compared With WT Mice

Fluorescence-activated cell sorting analysis revealed a significant increase in the percentage of CD45.2$^+$ cells in *Winnie* compared with age- and sex-matched WT mice. Gating on CD45.2$^+$ MHC II$^+$ and CD45.2$^+$ MHC II$^-$ viable cells showed that both of these populations were present in greater numbers in *Winnie* colons compared with WT (Fig. 3A), with CD45.2$^+$MHC II$^+$ cells increased from 3.9% in WT to 7.9% in *Winnie* ($P = 0.021$), whereas CD45.2$^+$ MHC II$^-$ cells increased from 1.8% in WT to 6.6% in *Winnie* ($P = 0.020$). Furthermore, a 4-fold increase in relative mRNA expression of *Cd45* was present in the colons of *Winnie* compared with WT mice (Fig. 3B), while *Cd45* and *MhcII* were below the detection limit in both 5-week-old WT and *Winnie* mice (data not shown).

Age-Dependent Microbiome Composition Is Distinct in *Winnie* vs WT Mice

We recently demonstrated that the intestinal microbiome of adult *Winnie* mice is clearly distinguishable from WT mice.[28] However, it is unclear if dysbiosis in *Winnie* mice is the result of colitis progression or if dysbiosis itself drives ensuing colonic inflammation. As such, we analyzed the microbiome of

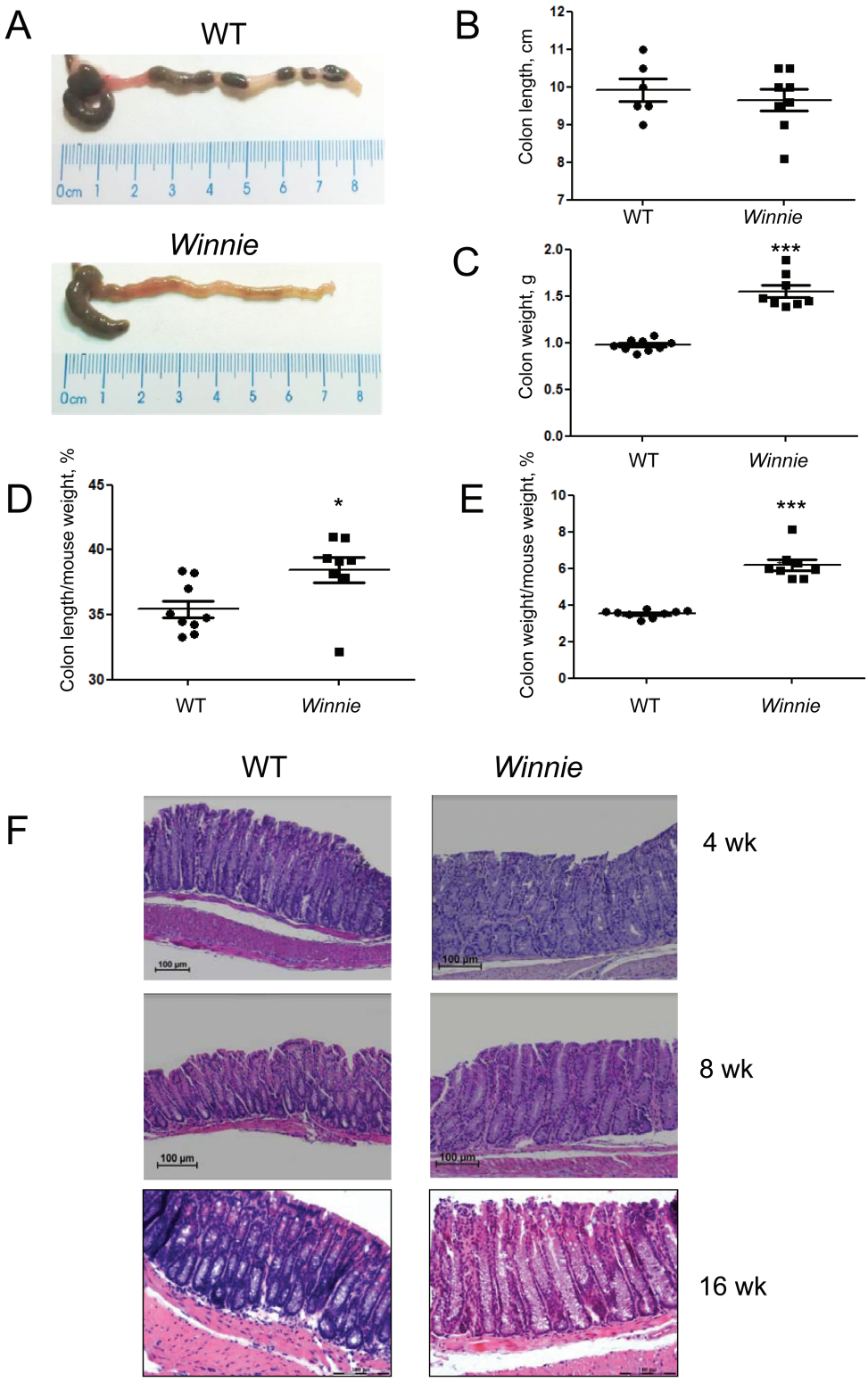


FIGURE 2. Aberrant morphologic and histologic features in colons from *Winnie* vs WT mice. Representative images of whole colons (A) and measurements of colon length (B) and colon weight (C) of 16-week-old *Winnie* and WT male mice. D and E, Colon length/mouse weight and colon weight/mice weight indices, respectively. F, Hematoxylin and eosin staining of distal colon sections shows abundant mucus-secreting goblet cells with moderate inflammation only in 16-week-old *Winnie* mice and evident signs of erosion within the intestinal epithelium compared with WT controls. Scale bar = 100 µm. No signs of inflammation are present in 4- and 8-week-old mice. $^{***}P < 0.001$; $^{*}P < 0.05$ (Student *t* test).

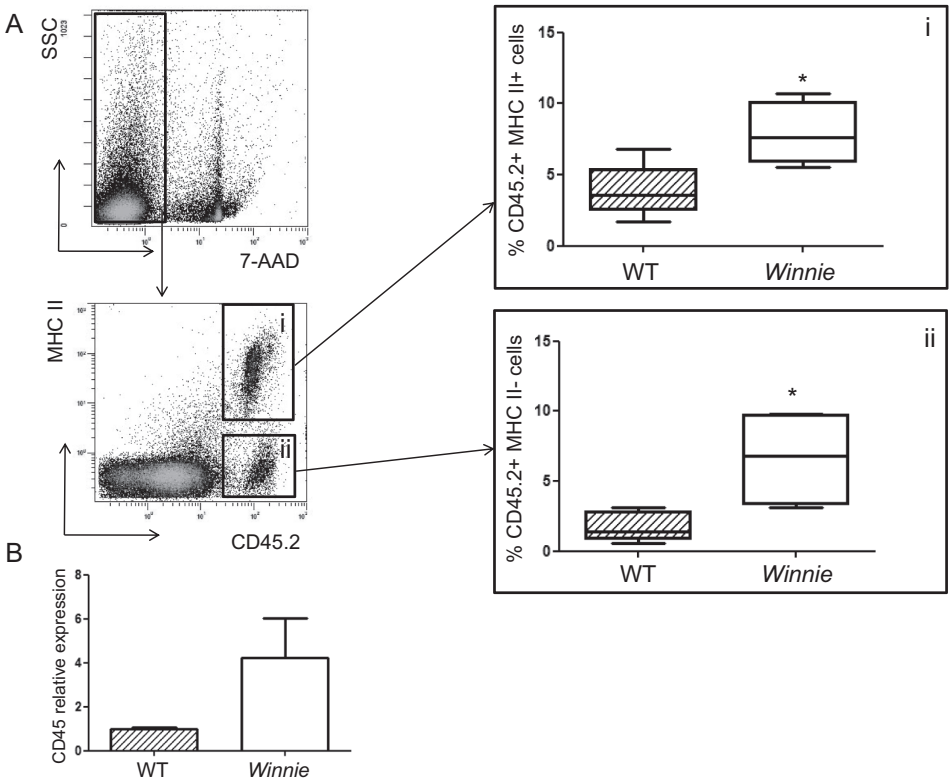


FIGURE 3. Increased immune cell infiltration within the lamina propria of *Winnie* compared with WT mice. A, Representative density plot for LP cells, with analyses performed on viable cells by excluding the 7-AAD$^+$ population. Graphs (right panels) represent percentages of CD45.2$^+$ MHC II$^+$ (i) and CD45.2$^+$ MHC II$^-$ (ii) cells in WT and *Winnie* mice. B, Relative expression of Cd45 in colons of *Winnie* and WT mice. Bars represent mean relative expression ± SEM (n = 3) for each genotype. *P < 0.05 (Student t test).

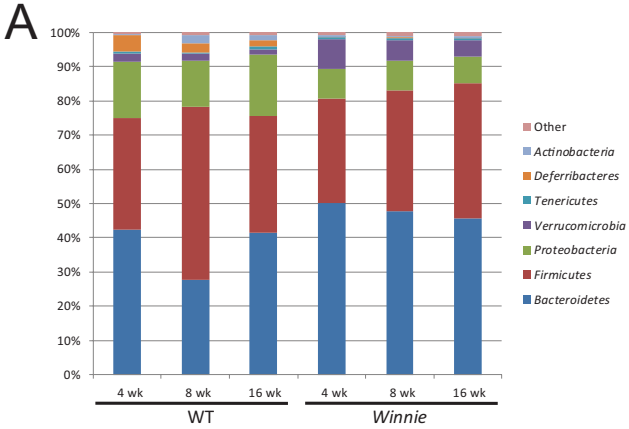
Winnie mice at different ages, from time of weaning to adult age, and compared the results obtained from WT mice to assess if dysbiosis was either the cause or effect of intestinal inflammation in this unique mouse strain.

Experimentally, we evaluated the microbiome composition in 4-, 8-, and 16-week-old WT vs *Winnie* mice. Compared with WT, *Winnie* mice showed the highest (P < 0.05) Shannon index value at 4 weeks of age (Supplementary Table 1). A similar trend was also observed at 8 and 16 weeks. The highest values of OTUs and Chao1 were evident in *Winnie* samples compared with WT. The only exception was for Chao1, which did not differ (P > 0.05) at 4 weeks of age. Three phylogeny-based β-diversity analyses, using the Unifrac distance metric, showed clustering of samples according to genotype (WT or *Winnie* mice) (Supplementary Fig. 1). Within the same genotype, samples taken at 4 weeks of age were clearly separate from the others (8 and 16 weeks), especially for WT samples.

In regards to the analysis based on phylum, abundance of Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia, Tenericutes, Deferribacteres, and Actinobacteria represented >99% of all 16S rDNA sequenced. Bacteroidetes and Firmicutes were the most representative phyla for both WT and *Winnie* mice. The relative abundance of Bacteroidetes was higher in *Winnie* compared with WT mice, particularly at 8 weeks of age. Firmicutes, however, did not differ between the 2 genotypes. In this regard, compared with 4 weeks of age, the relative amount of Firmicutes increased after 8 and 16 weeks of age for WT and *Winnie*, respectively (Fig. 4). The relative amount of Proteobacteria significantly differed among samples, especially for WT and *Winnie* at 16 weeks (17.85 vs 8.01%, respectively; P = 0.011). Verrucomicrobia were found at the highest relative levels in 4-week-old *Winnie* mice, after which relative abundance decreased with age. On the contrary, Deferribacteres was significantly lower in *Winnie* compared with WT samples.

In addition, compared with WT, *Winnie* mice showed the highest (P < 0.05) relative amount of *Alkaliphilus, Candidatus Blochmannia,* and *Caldilinea* genera in all weeks analyzed (Fig. 5). On the other hand, the presence of *Helicobacter*, *Odoribacter*, and *Lactobacillus* were mainly associated with WT samples. At 4 weeks, *Winnie* mice showed 3 other genera (*Dysgonomonas*, *Olivibacter*, and *Rubritalea*), which were all present in greater abundance than in WT mice. Compared with 4 weeks, the greatest differences in the profiles of genera were found in 8-week-old mice. *Bacteroides*, *Parabacteroides*, *Porphyromonas*, *Clostridium*, *Paraprevotella*, *Acholeplasma*,

A



B

	WT			*Winnie*		
	4 w	8 w	16 w	4 w	8 w	16 w
Bacteroidetes	42.42	27.68	41.52	50.06	47.63*	45.69
Firmicutes	32.5	50.53	34.03	30.51	35.4	39.36
Proteobacteria	16.42	13.61	17.85	8.82	8.83	8.01**
Verrucomicrobia	2.35	1.86	1.62	8.5**	5.94	4.62
Tenericutes	0.67	0.46	0.93	0.76	0.61	0.52
Deferribacteres	4.72	2.53	1.8	0.05	0.1***	0.19***
Actinobacteria	0.38	2.56	1.57	0.41	0.53	0.62
Other	0.54	0.77	0.68	0.88*	0.96	0.99

FIGURE 4. Changes in bacterial composition occur as early as 4 weeks in *Winnie* compared with WT mice. A, Average relative abundance (%) at 4, 8, and 16 weeks of age. The group "other" encompasses all phyla with relative abundance <0.01%. B, Values of relative abundance (%). Significance was calculated comparing bacterial abundance in *Winnie* vs WT at the respective time points. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ (Student *t* test).

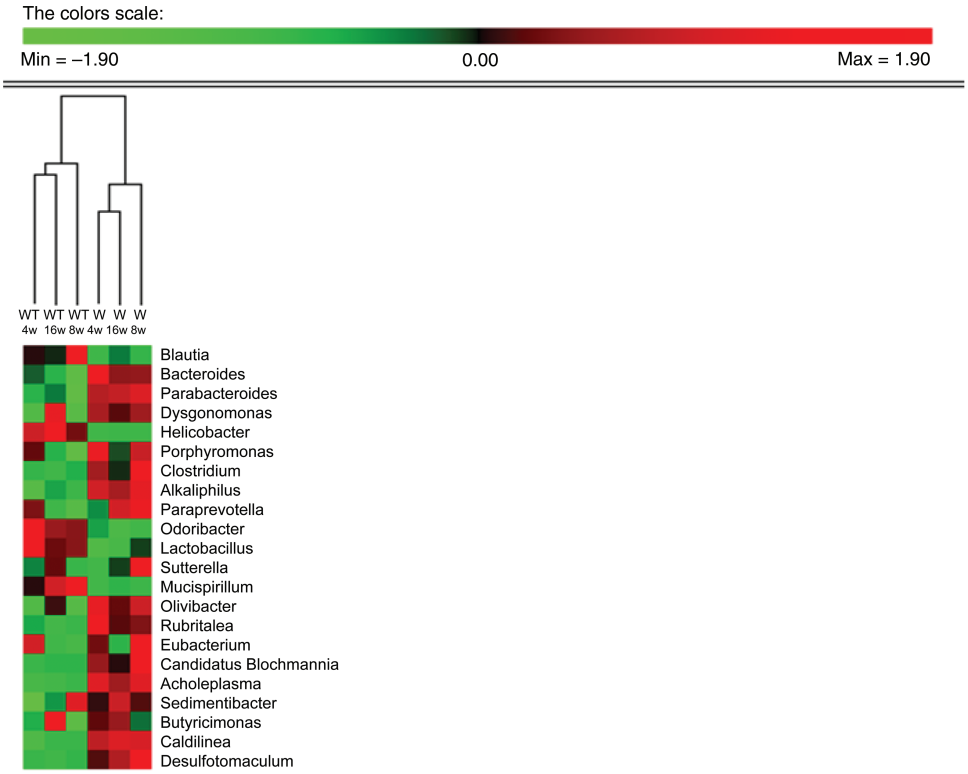


FIGURE 5. *Winnie* mice differ in the profile of genera compared with WT mice. Relative abundance (%) of total (16S rRNA) bacteria, differentially found ($P < 0.05$) at the genus level in fecal samples of WT and *Winnie* (W) mice at 4, 8, and 16 weeks of age.

Butyricimonas, and *Desulfotomaculum* were more abundant ($P < 0.05$) in *Winnie* compared with WT mice in 16-week-old mice and especially in 8-week-old mice. In contrast, *Blautia, Mucispirillum,* and *Sedimentibacter* were found to be more abundant in WT samples and increased in 8- and/or 16-week-old mice. Furthermore, we observed that the relative abundance of most bacterial genera changed with age, from 4 to 16 weeks of age. Surprisingly, the tendency to become more or less represented was similar for the vast majority of genera, independent from the host genotype. In particular, Figure 5 shows an increased presence of the *Mucispirillum* and *Caldilinea* genera in both experimental groups, whereas *Odoribacter* and *Bacteroides* decreased with age. Some bacterial genera, instead, showed an opposite tendency between the

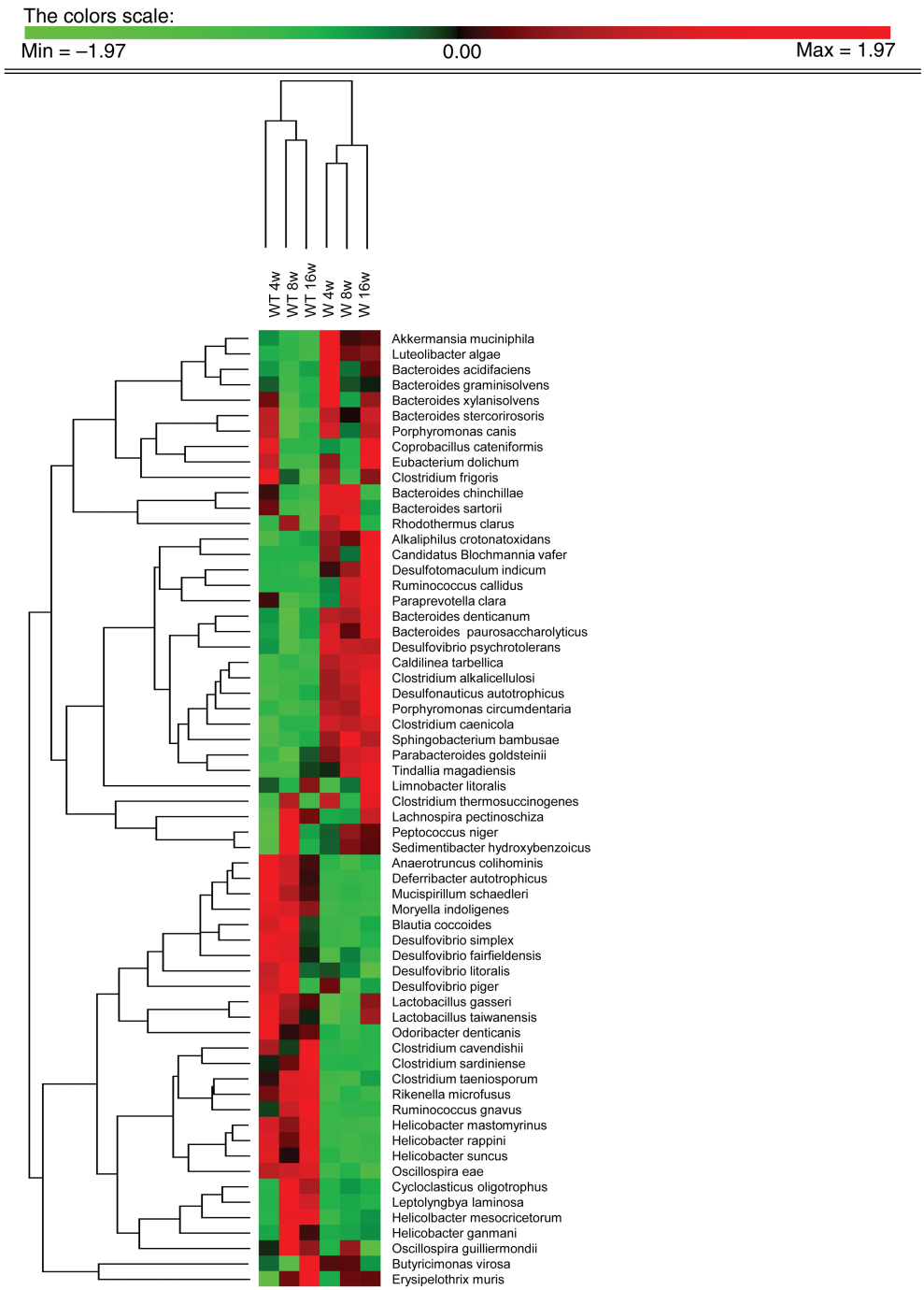


FIGURE 6. *Winnie* mice differ in the profile of species compared with WT mice. Relative abundance (%) of total (16S rRNA) bacteria, differentially found ($P < 0.05$) at the species level in fecal samples of *Winnie* (W) and WT mice at 4, 8, and 16 weeks of age.

2 genotypes. For example, *Parabacteroides* and *Lactobacillus* increased from 4 to 16 weeks of age in *Winnie* mice, but decreased in WT mice. This age-dependent tendency for decreased *Lactobacillus* in WT mice has been previously reported by Vemuri et al.[29]

Finally, in regards to species, the relative abundance of 62 species differed between samples (Fig. 6; Supplementary Table 2). Compared with WT, *Winnie* mice showed 9 bacterial species (*Akkermansia muciniphila, Alkaliphilus crotonatoxidans, Caldilinea tarbellica, Clostridium caenicola, Clostridium thermosuccinogenes, Desulfonauticus autotrophicus, Luteolibacter algae, Porphyromonas circumdentaria,* and *Tindallia magadiensis*) that were more abundant at 4 weeks of age ($P < 0.05$). All of the aforementioned species showed a

relative abundance that was always greater in *Winnie* than WT mice at 8 and 16 weeks of age. The highest differences in species profile (39 OTUs; $P < 0.05$) between genotypes were found in 8-week-old mice.

DISCUSSION

In this study, we compared the fecal microbiome of littermates (WT and *Winnie* mice) from the same mother(s) at 4, 8, and 16 weeks. Newborn pups were weaned immediately after the 4-week fecal material collection. The results of our study demonstrate that, even if delivered from the same mother and fed the same milk containing identical antibody repertoires, the specific genotype was the driving force for the unique microbiome footprint associated with either WT or *Winnie* mice.

Dysbiosis of the intestinal microbiome associated with chronic gut inflammation has become a major area of interest and research for IBD investigators. Abundance of microbial species seems to be a reliable indicator of IBD outcome; at the same time, persistence of intestinal dysbiosis can lead to disease recurrence, even in patients who successfully achieve remission. Maternal transmission has been indicated as the pivotal event for the onset of colonization of intestinal communities; for this reason, we investigated if the differences observed between the fecal microbiome of WT and *Winnie* littermates could still be observed in offspring from the same parents.

The *Winnie* murine model is a model of spontaneous and progressive UC displaying certain morphologic and microbial characteristics, which has been determined using conventional breeding approaches. Initially generating heterozygote breeders (*Winnie*$^{+/-}$) derived from C57BL/6 and homozygous *Winnie* mutant mice, we evaluated resulting littermate pups from the same mother(s). We first assessed the onset of the progressive UC-like colitis in the *Winnie* model by morphologic and histologic features, along with immune cell infiltrate of the large intestine. Strikingly, our fecal microbiota metagenomic analysis shows that *Winnie* offspring displayed significant dysbiosis as early as 4 weeks of age compared with their WT siblings/littermates the same age, which is similar to what has been reported at 12 weeks of age.[30] Overall, our data support a more dominant role for the host genotype in conferring dysbiosis onset compared with microbial transmission from the mother at birth. Of note, the possibility exists that analysis of microbiota derived from fecal samples may be more relevant if focused on mucus-associated bacteria present within the colon. However, due to the experimental design based on prospective microbiota analysis, it was not possible to collect samples from the same individual mouse at 4, 8 and 16 weeks.

An abundance of microbial species perfectly clustered, depending on host expression of the *Winnie* mutation, with the greatest significance at 8 weeks after weaning, when the immune machinery of the host is developing its immunoglobulin repertoire.[31] Bacteroidetes were mainly associated with the *Winnie* genotype. Previously, it was shown that Bacteroidetes establish a crucial interaction with the host at an early stage of life, and their colonization in germ-free mice affects immune system development.[32] Indeed, *Bacteroides* and *Prevotella*, 2 of the most abundant genera in the intestinal microbiome within the Bacteroidetes phylum, are associated with UC.[33] In contrast, a previous meta-analysis reported 9 studies (706 patients) published between 2002 and 2012 showing that the mean level of *Bacteroides* was significantly lower in CD and UC patients, especially in the active phase of disease.[34] The differences in *Bacteroides* may be assigned to individual species.

We also found that several species belonging to different genera (*Bacteroides*, *Parabacteroides*, *Porphyromonas*, *Paraprevotella,* and *Sphingobacterium*) of Bacteroidetes were mainly associated with the *Winnie* genotype, particularly in 8-week-old mice. The only 2 Bacteroidetes species reduced in *Winnie* compared with WT mice were *Odoribacter denticanis* and *Rikenella microfusus*. The controversial role of Bacteroidetes in the development of inflammatory intestinal diseases will be the focus of future studies in our lab and raises the possibility of promising therapies targeting mucosa-associated bacterial composition.

Within Firmicutes, some species (eg, *Alkaliphilus crotonatoxidans*, *Clostridium alkalicellulosi*) were associated with the *Winnie* genotype. On the contrary, other Firmicutes (eg, *Blautia coccoides*, *Oscillospira eae*, *Ruminococcus gnavus*) were less represented in *Winnie* compared with WT mice. Previously, dogs with chronic inflammatory enteropathy showed a lower amount of *Blautia* and *Ruminococcus* compared with healthy dogs.[35] *R. gnavus* showed a positive role in the intestinal microbiota, producing an antibacterial peptide that protects hosts against pathogens.[36]

Several studies have evaluated the influence of different genes on the intestinal microbiome and showed that NLRP6, an innate immune receptor, is important in suppressing the development of spontaneous colitis in *IL10*$^{-/-}$ mice.[37] Moreover, NLRP6 deficiency induces enrichment of *Akkermansia muciniphila* that can act as a pathobiont to promote colitis in genetically susceptible hosts. In this study, *Akkermansia muciniphila* was found to be more abundant in *Winnie* compared with WT mice at the time of weaning (4 weeks of age). This finding is somewhat surprising, as in IBD patients a decrease of this species has been observed.[38] *Akkermansia muciniphila* is a mucin-degrading bacterium; thus, in *Winnie* mice characterized by a compromised and less compact inner mucus layer, mucin availability could favor *Akkermansia muciniphila* outgrowth.[39,40] Indeed, *Winnie* mice are different from *Muc2* deficient mice, which are also characterized by acute spontaneous colitis and colorectal cancer development.[12,41] MUC2 is present in *Winnie*, but is not firmly compacted in a tight inner layer.[17] It is important to note that *Akkermansia muciniphila* abundance is reduced in older mice, when the inflammatory response becomes more evident ($P = 0.008$ in 4- vs 8-week-olds). These findings are in line with what has been previously reported.[27] Indeed, Schneeberger and colleagues correlated levels of

Akkermansia muciniphila with inflammatory markers and showed that these were inversely correlated.[42]

Interestingly, we found that the *Helicobacter* genus is mainly associated with WT mice, with *H. mastomyrinus* as a dominant species. *H. mastomyrinus* colonizes the liver of mice, but the pathogenic potential of this bacterium is unknown.[43] Indeed, the role of *Helicobacter* species in IBD is not yet fully understood and is somewhat controversial. *Helicobacter pylori* infection is negatively associated with IBD, regardless of ethnicity, age, and previous use of aminosalicylates and corticosteroids with antibiotics, influencing the magnitude of this association. Closely related bacteria, including *Enterohepatic Helicobacter* spp. (EHS) and *Campylobacter* spp., can also increase the risk of IBD.[43] It has also been suggested that *H. pylori* might exert an immunomodulatory effect in IBD.[44]

CONCLUSIONS

Overall, using the *Winnie* model of colitis, we demonstrate that alterations of genes, such as the *Muc2* mutation in this mouse strain, which impacts the maintenance of intestinal homeostasis, may result in early intestinal dysbiosis that is independent from maternal microbial transfer, including breastfeeding. Once established, differences between microbial communities persist and continue with age. Results from the present study were derived using heterozygote mothers; as such, future studies using mothers with different genetic backgrounds will be studied to further elucidate the influence of maternal microbial transfer on the offspring's microbiome. Our study has aided in providing a map of intestinal dysbiosis dependent on mutagenesis of *Muc2* that may be useful for future targeted interventional studies.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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